Arch. Environ. Contam. Toxicol. 25, 41–47 (1993)

ARCHIVES OF
Environmental Contamination and Toxicology

The Effect of Salinity on the Acute Toxicity of Cadmium to the Tropical, Estuarine, Hermaphroditic Fish, *Rivulus marmoratus*: A Comparison of Cd, Cu, and Zn Tolerance with *Fundulus heteroclitus*

Hui-Chen Lin and William A. Dunson

Department of Biology, 208 Mueller Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

Abstract. The mangrove-dwelling fish, *Rivulus marmoratus*, is the only vertebrate that is a synchronous, internally self-fertilizing hermaphrodite. This unique reproductive mode yields offspring with little genetic variation, which offers significant advantages for the use of this species in bioassays. We conducted acute (96 h) LC_{50} tests of Cd toxicity under four different water chemistry conditions, representing fresh water (low [Ca + Mg] and low [Na + K]), 14 ppt sea water simulated with Cl salts (high [Ca + Mg] and high [Na + K]) and two artificial conditions (high [Ca + Mg], low [Na + K] and low [Ca + Mg], high [Na + K]). Two replicates were conducted at different times for each of the four treatments and the results were very reproducible. The mean LC_{50}'s as mg total Cd/L were 2.96 (fresh water), 21.12 (high [Ca + Mg], high [Na + K]), 17.86 (high [Ca + Mg], low [Na + K]) and 12.67 (low [Ca + Mg], high [Na + K]). An additional test in 14 ppt sea water (made up from Instant Ocean™ salts) yielded a 96h LC_{50} of 24.48 mg Cd/L, and was thus similar to the high [Ca + Mg]-high [Na + K] treatment, despite some differences in anion and cation concentrations. The degree to which [Ca + Mg], [Na + K] and [Cl] interact to determine Cd toxicity is still unclear, although the role of [Cl] is likely to be equal to or greater than that of [Ca + Mg]. When all solutes are high, it is likely that the formation of a Cd complex with Cl (248 mM) leads to the observed reduction in Cd toxicity in comparison with hard fresh water, not the increased divalent [Ca + Mg] levels. Further tests are needed in which the various cations and anions are varied as independently as possible in order to determine their roles in affecting free [Cd], or interacting with the gills to reduce Cd toxicity.

Interstrain variation in Cd toxicity was marked; four strains of *R. marmoratus* were exposed to three levels of Cd (LC_{25}, LC_{50} and LC_{75} estimated from tests of high-[Ca + Mg], high-[Na + K] conditions). Strain DS was consistently the most sensitive, whereas strain NA was consistently the most resistant to Cd across the three Cd exposure levels. Thus we recommend use of a single strain to conduct toxicity tests, or randomization of strains across treatments.

Two replicate 96h LC_{50} tests of each of the three heavy metals, Cd, Cu and Zn, for *Rivulus marmoratus* were found to be similar to values for *Fundulus heteroclitus*, a standard estuarine bioassay fish. The mean LC_{50}'s of Cd, Cu and Zn at 14 ppt for *Rivulus* were 21.1, 1.4 and 147.9 mg/L, respectively, whereas those for *Fundulus* were 18.2, 1.7 and 129.5 mg/L. This similarity in toxicological response by two species from different biogeographic regions (tropical vs temperate) may be due to similar physiological and biochemical adaptations to the abiotically stressful estuary.

The atheriniform fish, *Rivulus marmoratus* (Cyprinodontidae) found in mangrove areas ranging from southern Florida throughout the Caribbean to central America is remarkable for its synchronous, internally self-fertilizing hermaphroditism (Harrington 1961). It is a unique vertebrate in that it reproduces sexually and uniparentally. It was completely homozygous for the 31 loci studied by Vrijenhoek (1985) and clonally stable for at least three generations as demonstrated by single-sequence DNA fingerprinting (Turner *et al.* 1990). Early studies (Kallman and Harrington 1964; Harrington and Kallman 1968) have shown the complete acceptance of tissue transplantation between parent and offspring, and between siblings. This highly inbred reproductive mode generates little genetic variation within each parental line and makes this species an ideal organism for use in bioassays (Davis 1986). *Rivulus marmoratus* matures within 2–3 months in small bowls and then produces eggs on a weekly basis throughout the year. Depending on the size and age of the fish and the amounts of food provided, *R. marmoratus* can produce up to 20 eggs per day (Lin and Dunson, unpublished data). *Rivulus marmoratus* has been used as a subject for teratogenic studies (Lindsey and Harrington 1972; Harrington and Crossman 1976; Davis 1988) and is one of a number of fish species that has been used for cancer research (Koenig and Chasar 1984; Park and Kim 1984; Thiyagarajah and Grizzle 1985, 1986). However, it has not been widely used as a bioassay species.

The toxicity of Cd to estuarine animals varies with salinity (Eisler 1971; Westernhagen *et al.* 1974; Jones 1975; Westernhagen and Dethlefsen 1975; Theede 1980; McLusky *et al.* 1986; Reardon and Harrell 1990; Forbes 1991). Usually, the LC_{50} increases as the salinity increases; one interpretation is that metals are simply less toxic at increased salinities due perhaps to interactive effects with ions such as Ca at the target

Table 1. Water chemistry during Cd tests. Concentrations in mg/L (mM in parentheses) are shown as the average of the two tests of the same treatment. The [Mg]/[Ca] ratios (as mM) of tests 1 to 6 and test 9 were set at 5–7.5, similar to that of sea water. [Cl] was estimated as [Na + K + 2(Ca + Mg)], except for test 9 which was a close simulation of sea water and contains anions other than Cl (Cl_{est} was thus calculated from the Cl/Na ratio of sea water). In the first column, high level of ions was abbreviated as H and low as L

Test no./Treatment	[Na]	[K]	[Ca]	[Mg]	$[Cl]_{est}$
1,2.H-Ca, H-Na + K	4224 (184)	231 (6)	157 (4)	614 (25)	8792 (248)
3,4.H-Ca, L-Na + K	12 (0.6)	1.2 (0.03)	178 (4)	618 (25)	2140 (60)
5,6.L-Ca, H-Na + K	4789 (203)	202 (5)	35 (0.8)	135 (6)	7842 (221)
7,8.L-Ca, L-Na + K	11 (0.5)	1.0 (0.02)	25 (0.6)	18 (0.8)	114 (3.2)
9. Instant Ocean™	4465 (194)	213 (6)	192 (5)	618 (25)	8037 (226)

tissue site. An alternative explanation is that the proportion of the total metal that is toxic (the "free" fraction) diminishes. This generally inverse relation of toxicity with salinity has been described for Zn and Pb (Jones 1975), for Cu and Ag (Coglianese 1982), and for 20 metals in 30 compounds (Dorfman 1977). These papers give very detailed discussions of the effects of varying water hardness and salinity on reducing metal toxicity. However, the interactive effects of non-toxic anions and cations with Cd toxicity over a range of Na/Ca ratios are relatively unexplored.

The purpose of this study was to evaluate the use of *R. marmoratus* as a bioindicator for tropical estuarine pollution. To do so, we (1) conducted acute (96 h) Cd tests under different water chemistry concentrations, (2) examined the role of non-toxic ions (Na + K, Ca + Mg, and Cl) in changing Cd toxicity, (3) examined interstrain variation in response to Cd, and (4) compared the 96h LC_{50}'s of Cd, Cu and Zn between *R. marmoratus* and *Fundulus heteroclitus*, a standard bioassay species for temperate zone estuarine ecotoxicological studies.

Materials and Methods

The Organisms

The fish stocks originated in Florida, USA, and Yucatan, Mexico, and were obtained from several suppliers. Five different inbred strains were obtained from Dr. K. Kallman at the Genetics Laboratory of the New York Aquarium; these came originally from Dr. R. Harrington, the first to describe self-fertilizing hermaphroditism in this species (Harrington 1961). These fish have been kept in captivity for 13–40 generations.

Our colony was established in May 1989, following standard procedures for cultivation (Koenig and Chasar 1984). Fish stocks and all experiments were maintained at 26–27°C and a photoperiod of 14L:10D. Adults were kept individually in 11.4 cm (4.5-in.) diameter stackable glass culture dishes (Carolina Biological) filled with 150–200 ml of approximately 12.5 ppt (≈33%) sea water made from a commercial synthetic mix (Instant Ocean™ powder). Newly hatched *Artemia* nauplii were fed to the fish at least 5 days a week to ensure good growth and reproduction, and the water was changed every 4–5 weeks. Eggs were incubated in dechlorinated tap water and transferred to 12.5 ppt sea water 5–10 days after hatching. Hatchlings about 4–6 weeks old (wet mass = 0.03–0.1 g; length = 11–18 mm) were used for testing.

Adult *Fundulus heteroclitus* were collected from an impoundment on Assateague Island, Virginia. Eggs and sperm were obtained by stripping the fish (three males and six females), as described by Costello *et al.* (1957). Fertilized eggs were incubated in 12.5 ppt sea water and hatching was stimulated by replacing this sea water with dechlorinated hard water. Hatchlings were maintained in 12.5 ppt sea water and fed with *Artemia* nauplii for 4–6 weeks before testing (wet mass = 0.02–0.13 g; length = 12–20 mm).

The Flow-Through Test System

This consisted of six bottles of 20 L stock solutions (one control and five levels of toxicant) connected to six 2.4 L plastic test chambers. For each 96 h acute toxicity test, 60 *R. marmoratus* were used; 10 in each container. The flow rate was controlled by a peristaltic pump (approximately 2–3 ml/min), with 90% replacement of solution in the container within 48 h (Sprague 1969).

Water samples taken at the beginning and the end of each test were filtered (0.1 μm pore size) and acidified for Cu and Zn tests only (see below for Cd protocol) with Ultrex™ (ultrapure reagent nitric acid) and analyzed using a Perkin-Elmer model 2280 atomic absorption spectrophotometer (air acetylene flame). These analyses gave total, non-particulate metal concentration.

Cadmium tests

To determine the effects of water cation concentrations on Cd toxicity, 96 h LC_{50} tests were conducted under five different water chemistry conditions. Four combinations of two levels of [Ca + Mg] and two levels of [Na + K] were tested and made to simulate 14 ppt sea water (≈40% SW, designated high [Ca + Mg], high [Na + K]), hard fresh water (low [Ca + Mg], low [Na + K]) and two artificial conditions (high [Ca + Mg], low [Na + K], and low [Ca + Mg], high [Na + K]). Note that concentrations of the divalent cations (Ca and Mg) and the univalent cations (Na and K) were each varied simultaneously, although the treatment designation (Table 1) refers only to the former member of each pair; with two replicate tests for each condition. Chloride salts (NaCl, KCl, $CaCl_2$, and $MgCl_2$) were used exclusively. One additional test was made, using Instant Ocean™ salts to make up approximately 40% sea water. Water chemistry parameters are summarized in Table 1. The toxicant used was cadmium sulfate ($3CdSO_4 \cdot 8H_2O$).

There was no difference in Cd concentrations of filtered and unfiltered samples from several pilot tests and no sign of decline in total Cd concentrations over time for any test conducted. Thus, we consequently acidified and measured Cd in samples without filtration. Several studies have also shown no loss of Cd concentration over time (Jackim *et al.* 1970; Eisler 1971; Eisler and Gardner 1973; Eilser and Hennekey 1977; Pascoe *et al.* 1986).

Interstrain Test

The four strains of *R. marmoratus* represented progeny of a single parental fish derived from Vero Beach, Florida (DS and NA), Miami, Florida (M) and Yucatan, Mexico (E). Fish were assigned to three levels of Cd concentrations, representing the average 96-h LC_{25}, LC_{50}, and LC_{75} of the three high Ca + Mg, high Na + K tests (tests 1, 2, and 9) estimated by the probit procedure, and a control containing Instant Ocean™ salts at 14 ppt (no cadmium). A total of 256 fish [(8 fish/container) × (2 containers/conc./strain) × (4 conc.) × (4 strains)] were to be used for this 10-day test. However, strain DS only had 14 individuals (instead of 16 as originally planned) for each of the three Cd concentrations. Also, one fish from strain M at the LC_{75} treatment level was lost at the beginning of the experiment. Within each strain, the fish were either from the same parent or from the same grandparent. There was no feeding during the test. Water samples were collected on days 0, 3, 7, and 10. The means ± SD of Cd concentrations were 20.66 ± 0.41, 23.88 ± 0.32, and 27.59 ± 0.15 mg total Cd/L for the LC_{25}, LC_{50}, and LC_{75} levels, respectively. Relatively small standard deviations indicated that the Cd concentrations were nearly constant.

Cd, Cu and Zn Tests

The same experimental flow-through device as described for the Cd toxicity tests was used to examine the toxicity of Cd, Cu, and Zn for *F. heteroclitus*, and Cu and Zn for *R. marmoratus*. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) and cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were used. Two tests were made for *R. marmoratus* and one for *F. heteroclitus* for each metal. Except for two Cd tests for *R. marmoratus*, all 96-h LC_{50} tests were performed in 40% sea water (≈14 ppt) made with Instant Ocean™ salts. Both Zn and Cu precipitated to varying degrees during the experiment. The samples were filtered at 0.1 μm (pore size) to remove particulates and acidified before analysis on an atomic absorption spectrophotometer.

Data Analyses

All LC_{50}'s were derived by the maximum likelihood estimate calculated with a SAS probit procedure (SAS Institute 1985) and are presented as mg total metal concentration per liter. We used the SAS GLM procedure (SAS Institute 1985) to derive a two-way analysis of variance (ANOVA) table and evaluate the effects of cations (Ca and Na) on the LC_{50}'s of Cd for *R. marmoratus*. The concentrations of Mg and K were kept at a fixed ratio with those of Ca and Na, respectively, and these ratios reflect those of seawater and hard fresh water conditions. This two-way ANOVA represents the effects of divalent and univalent cations. The 96-h LC_{50} values were normalized by a log transformation for the analysis of variance.

Life tables and survivor functions of BMDP 1L (BMDP 1990) were used to estimate mean survival time and to analyze interstrain variation. Since there is a predictable negative relationship between [Cd] and survival time, the different survivorship of fish among the three Cd levels was not of primary interest.

To compare the LC_{50}'s between two tests when their 95% fiducial limits did not overlap, the trimmed Spearman-Karber method (Hamilton et al. 1977) was employed, using the procedures suggested by the Water Quality Standards Handbook (EPA 1983). The required natural logarithm (ln) of LC_{50} and its variance were obtained and transformed from the SAS-probit output, designated as "mu" and "sigma" expressed as a $\log_{10}$ value.

Results

Tests of Cadmium Toxicity Under Various Water Chemistry Conditions

Both [Ca] and [Na] and their interaction had significant effects on Cd toxicity (Table 2). As electrolyte concentrations increased, the LC_{50} values increased; ie. Cd became less toxic (Figure 1). In tests 1 to 4 [Na + K] was varied, but [Ca + Mg] held at approximately the same high level. The four LC_{50}'s overlapped (Figure 1) and the numerical values of tests 1 to 4 were 23.70, 18.53, 16.93, and 18.79 mg/L, respectively. When [Na + K] was held at high levels and [Ca + Mg] varied (tests 1, 2, 5, and 6), the LC_{50}'s of tests 1 and 5 (the two most extreme of the four) were not statistically different ($p > 0.05$) using a trimmed Spearman-Karber method (EPA 1983). Tests 5 and 6 had estimated LC_{50} values of 11.73 and 13.60 mg/L, respectively. Tests conducted at low electrolyte concentrations had significantly lower LC_{50} values (3.03 and 2.89 mg/L for tests 7 and 8, respectively).

The overlaps in fiducial limits between tests 9 (LC_{50} = 24.48 mg/L) and tests 1 and 2 show the similarity in results when using Instant Ocean™ salts and similar overall concentrations of chloride salts only (Figure 1).

When Ca, Na, and Cl were plotted individually against the 96h LC_{50} values, the patterns were different (Figure 2). Tests 5 and 6 had higher LC_{50} values than tests 7 and 8 probably because of the higher Na (203 vs 0.5 mM in Table 1) and Cl levels (221 vs 3.2 mM). The potassium concentrations of these four tests were very low in comparison with those of Na and Cl. In contrast, tests 3 and 4 had higher LC_{50} values than tests 7 and

Table 2. Analysis of variance of effects of electrolyte levels on Cd toxicity. The effect of cation levels were confounded with that of Cl. Analyzed 96 h LC_{50} values were log transformed. df = degree of freedom, SS = Type III Sum of square

Source	df	SS	F value	p-value
Ca	1	0.571	235.97	0.0001
Na	1	0.286	117.91	0.0001
Ca × Na	1	0.158	65.32	0.0005
Error	5	0.012		

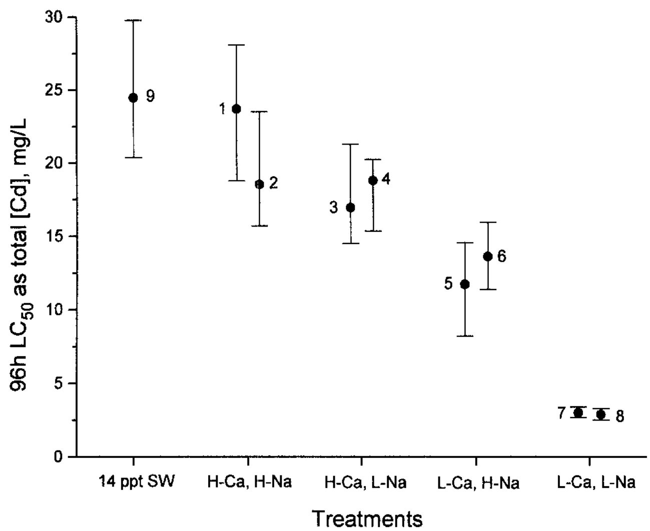

Fig. 1. The acute Cd toxicity for *R. marmoratus* expressed as concentration of total cadmium under various water chemistry conditions. The solid circle is the 96-h LC_{50} of each test, and the bars attached to it are the 95% fiducial limits. The numbers designate each test and correspond to those in the text and Table 1. H = high level, L = low level. SW = made with Instant Ocean™ sea water salts

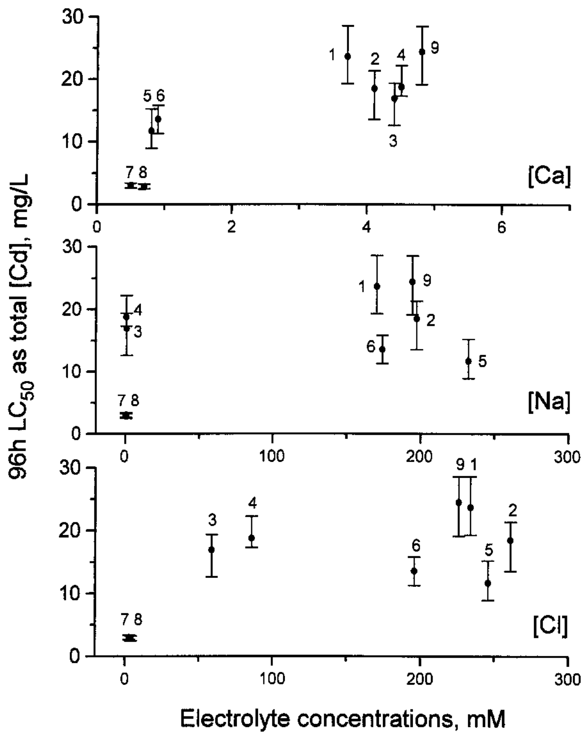

Fig. 2. The relation between the 96-h LC_{50} of Cd for *R. marmoratus* and concentration of each of the three non-toxic ions (Ca, Na, and Cl). The solid circle and the bars attached to it represent the LC_{50} and its 95% fiducial limits, respectively. The numbers are test numbers corresponding to Table 1 and Figure 1

Table 3. Variation of tolerance among four strains of *Rivulus marmoratus* held at three levels of Cd for 10 days. The Cd levels were the 96h LC_{25}, LC_{50}, and LC_{75} estimated from earlier tests using the pooled fish stocks. Values are mean survival time (days) ± SE

	Cd concentrations (mg/L)		
Strain	20.66 (LC_{25})	23.88 (LC_{50})	27.59 (LC_{75})
DS	3.86 ± 0.51	3.50 ± 0.14	2.64 ± 0.13
E	5.75 ± 0.76	5.50 ± 0.80	4.00 ± 0.51
M	9.25 ± 0.48	4.75 ± 0.58	2.93 ± 0.12
NA	9.25 ± 0.56	8.25 ± 0.84	4.75 ± 0.60

8 probably because of the higher Ca + Mg levels (29 vs 1.4 mM) *and* Cl levels (60 vs 3.2 mM).

Interstrain Variation in Cd Toxicity

Strain DS was the most sensitive, and NA the most resistant across the three Cd levels tested. The estimated mean survival time and its standard error for each strain are shown in Table 3. Based on times to death, strain NA was 1.8–2.4 times more resistant than strain DS. The responses of strains E and M were between these two extremes. Note that strain E was more sensitive than strain M at the LC_{25}, but became more resistant at the LC_{75}.

The variation in response among the four strains were all statistically significant at three different levels of Cd toxicity (LC_{25}, LC_{50}, and LC_{75}). The test statistics of the nonparametric linear rank tests in comparing the survivorship functions across the strains were all highly significant with a p-value ≤ 0.001, which supported the hypothesis that there was variation among strains in response to Cd toxicity (Table 4).

Table 4. Statistical tests for survivorship among the strains at three levels of Cd. The test statistic was derived from the Mantel-Cox test (Generalized-savage test)

Comparison	Test statistic	df	p-value
At LC_{25} (20.66 mg/L)	37.254	3	<0.0001
At LC_{50} (23.88 mg/L)	17.184	3	0.0006
At LC_{75} (27.59 mg/L)	21.342	3	0.0001

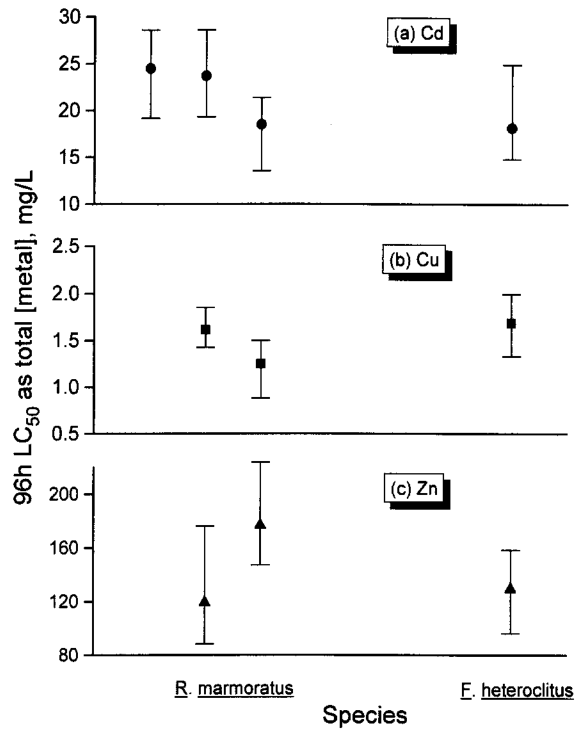

Fig. 3. The similarities of the 96-h LC_{50} and 95% fiducial limits of (a) Cd, (b) Cu, and (c) Zn for *Rivulus marmoratus* and *Fundulus heteroclitus*. The solid figure and the bars attached to it represent the LC_{50} and its 95% fiducial limits, respectively. The three Cd LC_{50} values for *R. marmoratus* (from left to right) were derived from tests 9, 1, and 2 (Table 1, Figure 1). Only tests 1 and 2 were conducted using a simulated sea water made up entirely with Cl salts. Instant Ocean™ salts were used for the rest of the tests. All tests were conducted at a salinity of 14 ppt and a temperature of 26°C. Fish were 1–2 months old (12–17 mm in length)

Comparison of Tolerance to Cd, Cu and Zn Tests with Fundulus

The LC_{50} values of the two species were similar; 95% fiducial limits overlapped and there was no statistical difference between them (Figure 3a). For *R. marmoratus* we used the LC_{50}'s of tests 1, 2, and 9 to compare with that of *F. heteroclitus*. Our estimate of the LC_{50} for *F. heteroclitus* was 18.2 mg/L with a 95% fiducial limit of 14.8 and 24.9 mg/L.

Although the LC_{50} of Cu for *F. heteroclitus* (1.69 mg/L) was numerically slightly higher than those of *R. marmoratus* (1.25 and 1.61 mg/L), the fiducial limits overlap with no statistical difference (Figure 3b).

The LC_{50}'s for Zn were also similar between *F. heteroclitus* and *R. marmoratus*. Our estimates of the LC_{50}'s were 129.5 mg/L for *F. heteroclitus* and 119.3 and 176.6 mg/L for *R. marmoratus*. Their fiducial limits overlapped and there was thus no statistical difference (Figure 3c).

Discussion

Cd Toxicity Under Various Water Chemistries

The toxicity of Cd was inversely related to the electrolyte concentrations. This study focused on the roles of non-toxic ions in reducing Cd toxicity by manipulating their concentrations while keeping the remaining electrolytes at defined levels. At low Ca + Mg levels, the LC_{50}'s of tests 5 and 6 were significantly higher than those of tests 7 and 8 (Figure 1), suggesting that [Na + K] has a bit less, if not the same, effect as that of Ca + Mg, on protecting fish from Cd poisoning. This interpretation is confounded by the fact that Cl was also much higher in tests 5 and 6, than in 7 and 8 (Table 1) and free Cd might be less in the former two tests due to complexation. In their study of the chemical speciation of Cd and other metals in 100% sea water (35 ppt), Zirino and Yamamoto (1972) found that the free Cd ion (Cd^{2+}), the most toxic form of Cd, can be as low as 2.5%. Sunda *et al.* (1978) provided an extensive discussion on the importance of free Cd ion concentration in their study of grass shrimp, *Palaemonetes pugio*. As the salinity increased from near 0 to 14 ppt, the percent free Cd decreases approximately from 63% to 10% (supposedly due to complexation with Cl), leading to an apparent decrease in Cd toxicity. There was an almost linear relationship with a negative slope between the log of the fraction of free ion to total Cd (designated as $\log([Cd^{2+}]/Cd_t)$) and salinity in their study. Thus, the general inverse relation between Cd toxicity and salinity is due to greater complexation of metals by Cl at higher salinity (Sunda *et al.* 1978; Engel and Fowler 1979). This can be tested by multiplying the percentage of free Cd times the measured LC_{50}'s at different salinities and observing if this essentially removes the salinity effect on toxicity. For our data, such a calculation indeed indicated that changes in free Cd were the major factor when comparing the decline in LC_{50} between tests 1, 2 vs 7, 8. Thus, the same amount of free metal may be present at the different LC_{50}'s characteristic of low and high salinities, despite a higher total metal concentration in the latter condition.

When other electrolytes were held at relatively low levels, [Ca + Mg] was correlated with reduced Cd toxicity (tests 3, 4, 7, and 8 in Table 1 and Figure 1). With a low stability of divalent cation-protein complex (compared to that of Mg) at the epithelium of fish gills (Szumski and Barton 1983), Ca has been suggested to compete with Cd for binding sites on the gills (Wright 1977, 1980; McCarty *et al.* 1978; Wright and Frain 1981; Wright *et al.* 1985; Pascoe *et al.* 1986). However, the [Cl] of tests 3 and 4 was a confounding variable. The difference in [Cl] (60 vs 3.2 mM) could lead to the formation of a greater degree of Cd complexation when [Cl] was higher. Indeed, using the values for proportion of free to total Cd at varying salinities provided by Sunda *et al.* (1978), Cl complexation can account for the majority, but not all, of the observed decrease in Cd toxicity.

Interstrain Variation in Cd Toxicity

Striking differences were found in Cd toxicity among strains; strain DS was apparently the most sensitive among the four tested. Strains E and M reversed sensitivity at low (LC_{25}) and high (LC_{75}) levels of Cd, although their responses at the LC_{50}'s were very close (with mean survival times of 5.50 days for E and 4.75 days for M). Thus the mortality curves of these two strains may share a similar inflection point, but with different slopes. This may also imply that the variation in sensitivity at different toxic levels can be a source of variation for estimating the spectrum of lethal concentrations and their 95% fiducial limits from probit analysis when a pool of strains is utilized for the study. Given the consistencies within strains observed here, a test group made up of a single strain is recommended for toxicity testing. Pilot studies should be conducted before a given strain is selected for toxicity tests. However, there is the logistic difficulty of rearing a large number of mature fish to produce hatchlings of the same strain, age and size. Thus, a randomized pool of various strains could be used for acute toxicity tests, especially if an estimate of the LC_{50} is the primary interest.

Comparisons with Fundulus heteroclitus*—Cd, Cu and Zn Tests*

Based on the 96h LC_{50}'s, *R. marmoratus* and *F. heteroclitus* were quite similar in their tolerance to Cd, Cu and Zn. Eisler and Hennekey (1977) reported the 96-h LC_{50} for *F. heteroclitus* to be 22 mg Cd/L at 20°C and 20 ppt salinity. Dorfman (1977) studied the effects of 20 metals in 30 compounds on *F. heteroclitus* and found the 96-h LC_{50}'s to be 36 and 23 mg Cd/L at 7.2 and 24 ppt salinity, respectively (20°C). Burton and Fisher (1990) reported the 48h LC_{50} for Cd (10 ppt, 20°C) in *F. heteroclitus* to be 44.4 mg Cd/L. Thus, our estimate of the 96-h LC_{50} for *Fundulus* of 18.2 mg Cd/L was analogous to those reported previously.

The estimates of 1.69 mg Cu/L for *F. heteroclitus* and 1.25 and 1.61 mg Cu/L for *R. marmoratus* were very comparable to other measurements of the 96h LC_{50}. Copper was the most toxic among the three metals studied, when comparing the LC_{50}'s expressed in molarity. Jackim et al. (1970) reported a 96-h LC_{50} for *F. heteroclitus* of 3.2 mg Cu/L; Dorfman (1977) had an estimate of 0.4–3.1 mg Cu/L at salinities ranging from 24.0 to 5.5 ppt.

The LC_{50}'s for Zn of *F. heteroclitus* and *R. marmoratus* in this study were very similar. However, Eisler and Hennekey (1977) reported a 96-h LC_{50} of 60 mg Zn/L for *F. heteroclitus*, while Dorfman (1977) reported 27.5 and 32.0 mg Zn/L at 23 and 6 ppt salinity respectively, using zinc sulfate. The 48-h LC_{50} of *F. heteroclitus* was estimated to be 96.5 mg/L by Burton and Fisher (1990). The concentrations reported here (129.5 mg/L for *F. heteroclitus* and an average of 147.9 mg/L for *R. marmoratus*) were much higher. The most obvious reason was the problem of precipitation which creates great difficulties in maintaining constant Zn levels. The water samples collected at the beginning and the end of the tests sometimes were very different in our study, especially those of high target concentrations. The differences among persons and/or laboratories in handling and collecting samples become more crucial when precipitation appears. In any case, we can reliably conclude that these two species were quite similar in their tolerance to Zn, regardless of the absolute value of the LC_{50}.

Free ion concentration is likely to be the most direct predictor of metal toxicity (Sunda and Guillard 1976; Sunda and Hanson 1987; Jonas 1989; Hughes and Poole 1991). Sunda and Hanson (1987) provide an extensive discussion of a technique for measuring free cupric ion concentration in sea water, and compare it with other techniques. They also summarize the literature on the proportion of inorganic copper species to total Cu; it was only about 1% (0.03–2%) across all the techniques listed. Among those methods available for estimating free metals in sea water, most of them still involve various assumptions, and calculation from total metal concentration and complexes (Hughes and Poole 1991). Sunda and Guillard (1976) stressed the importance of maintaining a stable pH in keeping free copper ion constant. However, the pH at the gill area tends to be lower due to the local release of carbon dioxide and this, in turn, facilitates the release of free metal from its precipitate or complex (Cusimano *et al.* 1986; Sorensen 1991). Thus, although the free ionic metal concentration in the testing solutions can be carefully controlled by the addition of different levels and kinds of chelators (Sunda *et al.* 1978; Oakden *et al.* 1984), it is not necessarily equivalent to the immediate metal concentration to which the fish gill is exposed (Pagenkopf 1983; Cusimano *et al.* 1986; Lauren and McDonald 1987; Playle *et al.* 1992). At the moment, there is no single method to directly determine the toxicity and bioavailability of the metals in solution. Measurement of total metal concentration is probably the least ambiguous way to present these data. Also, most of the LC_{50} values from previous studies were presented as total metals. Since only total metal concentrations were measured, the LC_{50}'s are likely to be over-estimates of the true effective levels of each metal.

One should not conclude that *Rivulus marmoratus* and *Fundulus heteroclitus* are necessarily toxicologically alike for other stresses. For example, *F. heteroclitus* was quite tolerant of low pH and has a 96-h LC_{50} (H_2SO_4) of pH 3.8–3.9 (Gonzalez *et al.* 1989, Dunson *et al.* in press). Other estuarine fish such as *Menidia beryllina* were considerably less tolerant (96h pH_{50} = 4.6). The estimate of the 96-h pH_{50} for *R. marmoratus* was 4.21, with 95% fiducial limits of 4.12–4.3 (unpublished data). *Rivulus marmoratus* is a promising species as an estuarine bioindicator. The naturally inbred genetic attributes of this species, its fast growth rate, and its simple husbandry are strong arguments for its adoption as a tropical estuarine bioassay species of choice. At present a much greater effort needs to be extended in screening a wide variety of xenobiotic toxins and natural stresses with the *Rivulus* system.

Acknowledgments. We could not have begun this study without the generous cooperation of Will Davis, Klaus Kallman, and Scott Ritchie in supplying animals and advice on husbandry. We are deeply appreciative of the assistance of P. DiGiacomo, A. Gaston, M. Glackin, J. Grazio, R. Hicks, K. Hinton, L. Jandrasits, A. Jones, R. Liberti, S. Longo, V. Marr, G. Miller, E. Moss, S. Poole, B. Rafferty, M. Rolle, D. Trego, and P. Zuchowski with maintenance of the fish and in assisting with some experiments. We also thank Dr. J. Derr and Dr. R. Hultquist for statistical advice and C. Rowe and M. Horne for their suggestions during the preparation of the manuscript. This work was supported by a NIH Biomedical Research Support Grant (430-01 PHS Biomed 8014) and the Biotechnology Institute of The Pennsylvania State University. It was also supported, in part, by EPA grant R817206-01 to W.A.D. However, these contents do not necessarily represent the policy of this agency, and one should not assume endorsement by the Federal Government. The Chincoteague National Wildlife Refuge kindly granted permission to collect *Fundulus* on Assateague Island.

References

BMDP Statistical Software (1990) BMDP statistical software manual, vol 2. University of California, Berkeley

Burton DT, Fisher DJ (1990) Acute toxicity of cadmium, copper, zinc, ammonia, 3,3′-dichlorobenzidine, 2,6-dichloro-4-nitroaniline, methylene chloride, and 2,4,6-trichlorophenol to juvenile grass shrimp and killifish. Bull Environ Contam Toxicol 44:776–783

Coglianese MP (1982) The effects of salinity on copper and silver toxicity to embryos of the Pacific oyster. Arch Environ Contam Toxicol 11:297–303

Costello DR, Davidson ME, Eggers A, Fox MH, Henley C (1957) Methods for obtaining and handling marine eggs and embryos. Marine Biological Laboratory, Woods Hole, MA, pp 228–233

Cusimano RF, Brakke DF, Chapman GA (1986) Effects of pH on the toxicities of cadmium, copper and zinc to steelhead trout (*Salmo gairdneri*). Can J Fish Aquat Sci 43:1497–1503

Davis WP (1986) The role of *Rivulus marmoratus* in research on aquatic pollutants. J Am Killifish Assoc 19(1):70–80

——— (1988) Reproductive and developmental responses in the self-fertilizing fish, *Rivulus marmoratus*, induced by the plasticizer, di-n-butylphthalate. Environ Biol Fish 21(2):81–90

Dorfman D (1977) Tolerance of *Fundulus heteroclitus* to different metals in salt waters. Bull NJ Acad Sci 22(2):21–23

Dunson W, Fricano P, Sadinski W (1993) Direct and indirect effects of abiotic factors on a salt marsh fish assemblage. Wetlands 13 (in press)

Eisler R (1971) Cadmium poisoning in *Fundulus heteroclitus* (Pisces: Cyprinodontidae) and other marine organisms. J Fish Res Board Can 28:1225–1234

Eisler R, Gardner GR (1973) Acute toxicology to an estuarine teleost of mixtures of cadmium, copper and zinc salts. J Fish Biol 5:131–142

Eisler R, Hennekey RJ (1977) Acute toxicities of Cd^{2+}, Cr^{+6}, Hg^{2+}, Ni^{2+}, and Zn^{2+} to estuarine macrofauna. Arch Environ Contam Toxicol 6:315–323

Engel DW, Fowler BA (1979) Factors influencing cadmium accumulation and its toxicity to marine organisms. Environ Health Perspectives 28:81–88

Environmental Protection Agency (1983) Water quality standards handbook. Office of Water Regulations and Standards, USEPA, Washington, DC, pp B1–B3

Forbes VE (1991) Response of *Hydrobia ventrosa* (Montagu) to environmental stress: Effects of salinity fluctuations and cadmium exposure on growth. Funct Ecol 5:642–648

Gonzalez R, Mason C, Dunson W (1989) Anomalous tolerance to low pH in the estuarine killifish, *Fundulus heteroclitus*. Comp Biochem Physiol 94C:169–172

Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol 11:714–719

Harrington RW (1961) Oviparous hermaphroditic fish with internal self-fertilization. Science 134:1749–1750

Harrington RW, Crossman RA (1976) Temperature-induced meristic variation among three homozygous genotypes (clones) of the self-fertilizing fish *Rivulus marmoratus*. Can J Zool 54:1143–1155

Harrington RW, Kallman KD (1968) The homozygosity of clones of the self fertilizing hermaphroditic fish *Rivulus marmoratus* Poey (Cyprinodontidae, Atheriniformes). Am Nat 102:337–343

Hughes MN, Poole RK (1991) Metal speciation and microbial growth-the hard (and soft) facts. J Gen Microbiol 137:725–734

Jackim E, Hamlin M, Sonis S (1970) Effects of metal poisoning in five liver enzymes in the killifish (*Fundulus heteroclitus*). J Fish Res Board Can 27:383–390

Jonas RB (1989) Acute copper and cupric ion toxicity in an estuarine microbial community. Appl Environ Microbiol 55(1):43–49

Jones MB (1975) Synergistic effects of salinity, temperature and heavy metals on mortality and osmoregulation in marine and estuarine isopods (Crustacea). Mar Biol 30:13–20

Kallman KD, Harrington RW (1964) Evidence for the existence of homozygous clones in the self fertilizing hermaphroditic teleost (*Rivulus marmoratus*). Biol Bull 126(1):101–114

Koenig CC, Chasar MP (1984) Usefulness of the hermaphroditic marine fish, (*Rivulus marmoratus*), in carcinogenicity testing. Natl Cancer Inst Monogr 65:15–33

Lauren DJ, McDonald DG (1987) Acclimation to copper by rainbow trout, *Salmo gairdneri*: Biochemistry. Can J Fish Aquat Sci 44:105–111

Lindsey CC, Harrington RW (1972) Extreme vertebral variation induced by temperature in a homozygous clone of the self-fertilizing cyprinodontid fish *Rivulus marmoratus*. Can J Zool 50:753–763

McCarty LS, Henry JAC, Houston AH (1978) Toxicity of cadmium to goldfish, *Carassius auratus*, in hard and soft water. J Fish Res Board Can 35:35–42

McLusky DS, Bryant V, Campbell R (1986) The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. Oceanogr Mar Biol Annu Rev 24:481–520

Oakden JM, Oliver JS, Flegal AR (1984) EDTA chelation and zinc antagonism with cadmium in sediment: effects on the behavior and mortality of two infaunal amphipods. Mar Biol 84:125–130

Pagenkopf GK (1983) Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ Sci Technol 17(6):342–352

Park E, Kim DS (1984) Hepatocarcinogenicity of diethylnitrosamine to the self-fertilizing hermaphroditic fish *Rivulus marmoratus* (Teleostomi: Cyprinodontidae). J Natl Cancer Inst 73(4):871–876

Pascoe D, Evans SA, Woodworth J (1986) Heavy metal toxicity to fish and the influence of water hardness. Arch Environ Contam Toxicol 15:481–487

Playle RC, Gensemer RW, Dixon DG (1992) Copper accumulation on gills of fathead minnows: influence of water hardness, complexation and pH of the gill microenvironment. Environ Toxicol Chem 11:381–391

Reardon IS, Harrell RM (1990) Acute toxicity of formalin and copper sulfate to striped bass fingerlings held in varying salinities. Aquaculture 87:255–270

SAS Institute (1985) SAS user's guide: Statistics, version 5 edition. SAS Inst Inc, Cary, NC

Sorensen EMB (1991) Metal poisoning in fish. CRC Press, Ann Arbor, MI

Sprague JB (1969) Measurement of pollutant toxicity to fish I. Bioassay methods for acute toxicity. Wat Res 3:793–821

Sunda W, Guillard RRL (1976) The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J Mar Res 34:511–529

Sunda WG, Engel DW, Thuotte RM (1978) Effect of chemical speciation on toxicity of cadmium to grass shrimp, *Palaemonetes pugio*: importance of free cadmium ion. Environ Sci Technol 12(4):409–413

Sunda WG, Hanson AK (1987) Measurement of free cupric concentration in sea water by a ligand competition technique involving copper sorption onto C_{18} SEP-PAK cartridges. Limnol Oceanogr 32(3):537–551

Szumski DS, Barton DA (1983) Development of a mechanistic model of acute heavy metal toxicity. In: Bishop WE, Cardwell RD, Heidolph BB (eds) Aquatic toxicology and hazard assessment: Sixth symposium, ASTM STP 802. American Society for Testing and Materials, Philadelphia, pp 42–72

Theede H (1980) Physiological responses of estuarine animals to cadmium pollution. Helgo Wiss Meeresunters 33:26–35

Thiyagarajah A, Grizzle JM (1985) Pathology of diethylnitrosamine toxicity in the fish *Rivulus marmoratus*. J Environ Pathol Toxicol Oncol 6(2):219–232

Thiyagarajah A, Grizzle JM (1986) Nuclear glycogen and lipid in hepatocytes of a fish, *Rivulus ocellatus marmoratus*. Can J Zool 64:2868–2870

Turner BJ, Elder JF, Laughlin TF, Davis WP (1990) Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting. Proc Natl Acad Sci 87:5653–5657

Vrijenhoek RJ (1985) Homozygosity and interstrain variation in the self-fertilizing hermaphroditic fish, *Rivulus marmoratus*. J Hered 76:82–84

Westernhagen H, Dethlefsen V (1975) Combined effects of cadmium and salinity on development and survival of flounder eggs. J Mar Biol Assoc UK 55:945–957

Westernhagen H, Dethlefsen V, Sperling K-R (1974) Combined effects of cadmium and salinity on development and survival of herring eggs. Helgo Wiss Meeresunters 26:416–433

Wright DA (1977) The effect of calcium on cadmium uptake by shore crab, *Carcinus maenas*. J Exp Biol 67:163–173

———(1980) Cadmium and calcium interactions in the freshwater amphipod *Gammarus pulex*. Freshwat Biol 10:123–133

Wright DA, Frain JW (1981) The effect of calcium on cadmium toxicity in the freshwater amphipod, *Gammarus pulex* (L.). Arch Environ Contam Toxicol 10:321–328

Wright DA, Meteyer MJ, Martin FD (1985) Effect of calcium on cadmium uptake and toxicity in larvae and juveniles of striped bass (*Morone saxatilis*). Bull Environ Contam Toxicol 34:196–204

Zirino A, Yamamoto S (1972) A pH-dependent model for the chemical speciation of copper, zinc, cadmium, and lead in sea water. Limnol Oceanogr 17:662–671

Revised manuscript received January 14, 1993.